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Real-Time Imaging of Cell Behaviors in Living Organisms by a Mitochondria Targeting AIE Fluorogen

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Real-time visualizing behaviors of cell populations within living multicellular organisms is of great value to life science but challenging due to the lack of ideal probes. In this work, we report a biocompatible fluorogen, azide-functionalized tetraphenylethene pyridinium (TPE-PyN₃), for noninvasive imaging and sensing within living systems. TPE-PyN₃ exhibits unique aggregation-induced emission (AIE) attributes and high affinity to mitochondria, enabling it to achieve specific mitochondrial imaging and long-term cellular observing with excellent photostability both *in vitro* and *in vivo*. The high membrane penetrability of TPE-PyN₃ allows all of the cells within the living zebrafish embryos to be morphologically visualized and reconstructed in three-dimensions. Moreover, TPE-PyN₃ is capable of indicating cell apoptosis because of its sensitivity to the change of mitochondrial membrane potential. Our findings presented here provide a simple and noninvasive tool for studying behaviors of cell populations *in vivo* for the first time by a small-molecule AIE probe.

1. Introduction

Multicellular organisms are communities of cells that organize together to carry out complex processes of life.^[1, 2] By cooperation and specialization, various cells combine to form a coordinated

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system with more capabilities than any of its component parts. In order to study the biological mechanisms of living systems, *in vitro* cell culture-based methods have been extensively employed. However, owing to the separation from native environments, analysis of cells *in vitro* often does not represent the real circumstances *in vivo*. The findings *in vitro* are thus not applicable for direct extrapolation to the living organism.^[3] Moreover, science in the fields of modern developmental, cellular, and systems biology has evolved beyond understanding microstructures and isolated activities to the research of their complicated interrelationships within organisms.^[4] Observing the biological processes on the system level is thus of great scientific value.

Fluorescent probes have revolutionized the way in which researchers observe life processes in terms of sensitivity and simplicity.^[5, 6] Although cultured cell visualization *in vitro* using fluorescent probes has been tremendously advanced, their application in complex organisms remains challenging. For monitoring biological processes such as embryo development, tumorigenesis, and tissue morphogenesis in model organisms, it is often desirable to observe behaviors of cell populations within the living system. However, in most multicellular organisms, extracellular matrix and cell junctions^[7, 8] that provide structural barriers to the surrounding cells are impenetrable to common fluorophores due to their poor penetrability. It is difficult to image a large cell population within the whole organism using conventional fluorophores unless it is fixed, permeabilized or injected.^[9, 10] The need for cell imaging *in vivo* has promoted the emergence of methods such as genetically modified organisms expressing fluorescent proteins and quantum dots.^[11-13] Most of them, however, require sophisticated platforms and nontrivial manipulations. Worse still, these membrane-impermeable probes were generally injected into embryos, which can cause severe damage to delicate organisms.^[14] Another challenge is monitoring the dynamic processes in a long period of time. Under physiological conditions, conventional fluorescent dyes can hardly be retained inside organisms over a large time span and can be quickly bleached under the continual irradiation from fluorescent microscopes.^[15, 16]

Given these limitations, ideal probes for visualizing cell behaviors in living systems are highly desired.

Our group has discovered a group of luminogens which are nonemissive when molecularly dissolved but highly fluorescent when aggregate. We termed aggregation-induced emission (AIE) for this unique photophysical phenomenon and proposed restriction of intramolecular rotation (RIR) as its main cause.^[17, 18] Thanks to the unique AIE attributes, a variety of AIE molecules have been designed and utilized in diverse biological applications including organelles probing and cell tracing.^[19-23] Although these fluorophores offer noticeable advantages over conventional dyes, vital staining and *in situ* imaging of cells in living systems using AIE fluorophore has still yet to be explored.

In this contribution, we focus on a yellow fluorogen named azide-functionalized tetraphenylethene pyridinium (TPE-PyN₃) after screening our library of dozens of AIE molecules both *in vitro* and *in vivo*. TPE-PyN₃ was previously reported as a precursor molecule of the fluorescent probe for caspase activities. TPE-PyN₃ can be synthesized by the incorporation of pyridinium units into tetraphenylethene (TPE) through vinyl functionality.^[24] We uncover that TPE-PyN₃ exhibits high specificity to cellular mitochondria and can track the living cells for a long time with excellent photostability. In addition, its novel applications for cell populations imaging, high-quality 3D tissue visualization, and cell apoptosis sensing *in vivo* have also been successfully unveiled, indicating that TPE-PyN₃ may serve as a nonvasive tool for studying behaviors of cells within living organisms in a wide range of research fields.

2. Results and Discussion

2. 1. Cellular Mitochondria Targeting

TPE-PyN₃, which consists of a pyridinium group and a typical AIE unit-tetraphenylethene (TPE) (**Chart 1**), is AIE-active: It has an absorption maximum at 405 nm and emits weakly with a maximum peak at 636 nm in DMSO. In a mixture of DMSO-H₂O (v : v = 1 : 99), TPE-PyN₃ molecules cluster into nanoaggregates and emit strong yellow fluorescence with a quantum yield of 9.9%.^[24] The purity and identity of the molecule were verified by NMR and HR-MS (MALDI-TOF), prior to the use in the experiments. Biocompatibility of TPE-PyN₃ was evaluated by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cell viability was not significantly affected when up to 3 μ M TPE-PyN₃ was added (**Figure S1**), indicative of its low cytotoxicity.

After incubating with cultured cells as short as 5 min, TPE-PyN₃ can selectively accumulate in cellular mitochondria and emits strong yellow fluorescence (**Figure 1A, S2A**). The specificity to mitochondria was verified by costaining with a commercially available mitochondria targeting probe, MitoTracker red FM (MT, structure shown in **Chart S1**). Pearson's correlation coefficient (R_r ; from +1 to -1), which measures the linear association of two variables, was used to analyze the scope of distribution between two fluorescent channels. Fluorescent images of cervical cancer HeLa cells and non-cancerous fibroblast 3T3 cells after co-staining show that TPE-PyN₃ is co-localized with MT in mitochondria (**Figure 1, S2**) with R_r = 0.96 (HeLa) and 0.95 (3T3), respectively. These results suggest that TPE-PyN₃ is highly specific to mitochondria and can serve as a fluorescent bioprobe for cellular mitochondrial morphology imaging.

2. 2. Long-Term Fluorescent Cellular Tracing with Superior Photostability

When the staining time of TPE-PyN₃ was increased to 1 hour at a concentration of 3 μ M, the fluorophores tended to form bigger aggregates with strong emission in the cytoplasm (**Figure 2**). The aggregates would not diffuse from the cytoplasm to extracellular media after the excess dye were washed away. This special property prompted us to investigate its feasibility in cells tracking. We first

evaluated the specificity of TPE-PyN₃ in labeled cells under physiological conditions. HeLa cells stained with TPE-PyN₃ were co-cultured with unstained 3T3 in fresh culture media. After co-culturing overnight, the fluorescent aggregates were kept inside the HeLa cells, while the untreated 3T3 cells were still non-emissive (**Figure S3**), indicating that the internalized TPE-PyN₃ did not transfer from stained cells to unstained cells during co-culturing.

Next, we evaluated the retention time of TPE-PyN₃ in viable cells. We compared the performance of TPE-PyN₃ with that of 5-chloromethylfluorescein diacetate (CMFDA), a well-known commercially available dye for long-term cell labeling by chemically reacting with thiols on proteins and peptides to form aldehyde-fixable conjugates (structure shown in **Chart S2**). Cultured HeLa cells were stained with TPE-PyN₃ or CMFDA and sub-cultured every 24 hours. The fluorescence from labeled cells was monitored during each generation. To our delight, the fluorescent signal from TPE-PyN₃ was still well-retained in living cells for multiple generations, which was comparable to that of the CMFDA (**Figure 2**). Most cell-permeant fluorescent dyes, including fluorescein diacetate and carboxyfluorescein diacetate, are retained in living cells for no more than a few hours at physiological conditions.^[10] Furthermore, unlike CMFDA, the emission retention of TPE-PyN₃ was caused by forming aggregates rather than chemically linking to the intracellular biomacromolecules, which may have less adverse effect on the biology of cells.

For long-term observation, the fluorescent probe should be photostable in the biological environment. The photostability of TPE-PyN₃ and CMFDA stained HeLa cells was assessed by sequential scanning with confocal microscope. As shown in **movie 1**, TPE-PyN₃ remained brightly fluorescent for more than 1100 seconds and the signal attenuation was only about 30% after 50 scans with a total irradiation time of ~ 11 min (**Figure 3**). By contrast, the fluorescence of CMFDA was almost invisible just after 562 seconds (**Figure S4, movie 2**) and the signal attenuation was almost

90% after 50 scans (**Figure 3**). The excellent photostability of TPE-PyN₃ may due to its special AIE property: Although the outermost layer of molecules may be photobleached after irradiation, the condensed aggregates can prevent the interior molecules from photobleaching and photo-oxidating by blocking oxygen diffusion into the aggregates, resulting in superior photostability. For CMFDA, it mainly exists as single molecular form and thus can be easily bleached when exposed to excitation light, which greatly compromise its stability.

2. 3. Imaging of Cell Morphology in living Zebrafish Embryos

We envisaged that the remarkable properties of TPE-PyN₃ may be applied to imaging *in vivo*. Zebrafish (*Danio rerio*), a widely-used vertebrate model organism in scientific research,^[25, 26] was utilized to assess the spatiotemporal performance of TPE-PyN₃ *in vivo*. Live zebrafish embryos at 72-hour stage were incubated with 3 μ M TPE-PyN₃ for 2 hour and no marked differences of the mortality rate between the treatment and control group is observed, suggesting a low toxicity of TPE-PyN₃ to zebrafish embryos under the above-mentioned condition (detailed toxicity assessment data shown in **Figure S5**). After vital staining, TPE-PyN₃ covered the surface of whole embryo with strong fluorescence (**Figure 4**). Notably, all of the surface cells were strongly luminescent, indicating TPE-PyN₃ can penetrate through extracellular matrix and cell membrane and retain strong emission inside living cells. It is noteworthy that TPE-PyN₃ mainly stains the mitochondria in cells. Nonemissive nucleus region and boundary in each cell suggests that TPE-PyN₃ does not localize in cell membrane and nucleus. This enables it to precisely reveal the shape, boundary and location of each cell as well as its mitochondria and nucleus morphology within living embryos (**Figure 4B, H**). Additionally, in line with the results *in vitro*, living zebrafish embryo stained by TPE-PyN₃ remain fluoresce brightly and the cellular morphology can still be clearly visualized even after 60 hours under physiological conditions (**Figure 5**), indicative of the excellent retention behaviors and stability of TPE-PyN₃ *in*

in vivo. These properties can be of great value, especially in investigating the cellular morphology and behaviors involved in cell differentiation, growth and organogenesis in a long period of time.

Inspired by its wide spatial distribution, we next sought to evaluate whether TPE-PyN₃ could enable visualization of the whole sample in three-dimensions (3D). Serially laser scanning by confocal microscope was used to investigate the morphology of the living zebrafish embryo. We observed that the excellent photostability of TPE-PyN₃ enables it to withstand repeated laser illumination and a 3D morphological visualization of the embryo was successfully reconstructed. As shown in **Figure 4D**, G (corresponding to the bright-field images of **Figure 4C**, F), the high physical coverage and strong emission of TPE-PyN₃ render the 3D reconstruction of zebrafish embryo with high resolution. Tissue structures and cell morphology within the living embryo can be clearly observed and measured in a 3D visualization (**movie 3**). The reconstructed images allow various length, depth measurements to be made, and specific views at different cross-sections (**Figure 4E**) can be extracted to reveal structures of interest in the specimen. Due to the decayed excitation strength at deeper biological tissues,^[27-29] the confocal microscopy combined with TPE-PyN₃ labeling can only allow whole superficial imaging of the embryo.

2. 4. Real-Time Sensing of Cell Apoptosis *in vivo*

Since TPE-PyN₃ mainly localized in the mitochondria of embryonic cells, we further explored its value for bio-application at the organelle level. Energized mitochondria in healthy cell maintain a proton gradient the bilayer with large membrane potential ($\Delta\Psi_m$) of around -180 mV.^[30] Disruption of mitochondrial $\Delta\Psi_m$ is a distinctive feature of the early stages of cell apoptosis.^[31] To investigate the performance of TPE-PyN₃ to the change of mitochondrial $\Delta\Psi_m$, cadmium sulfate or staurosporine, two apoptosis-inducing chemicals with distinct mechanisms^[32, 33] were used to treat living zebrafish embryos stained with TPE-PyN₃. Surprisingly, in most of the cells, we observed that the yellow

fluorescence of TPE-PyN₃ almost disappear either in 100 μ M cadmium sulfate (**Figure 6B**, E) or 1 μ M staurosporine (**Figure 6C**, F) treated group, leaving only weak green autofluorescence. By contrast, mitochondria in the control group remained highly emissive (**Figure 6D**) and the morphologies of all cells can still be clearly visualized (**Figure 6A**).

Restriction of intramolecular motions (RIM) is proposed as the main cause of the AIE phenomenon.^[18, 34] The RIM effect of TPE-PyN₃ in cells may be mainly caused by the accumulation of cationic TPE-PyN₃ nanoaggregates to electronegative interior of the mitochondria. During cell apoptosis, the decreased mitochondrial $\Delta\Psi_m$ would lead to the diffusion of TPE-PyN₃ from intracellular mitochondria to extracellular medium due to the concentration gradient, resulting in the remarkable fluorescence “turn-off” response. The distinct difference of emission in two groups and the long retention time of TPE-PyN₃ make it an excellent tool for real-time monitoring cell apoptosis in living systems, which would be of great value for screening toxicity of chemicals, evaluating effects of pharmacological agents, as well as for studying apoptosis-related diseases.

3. Conclusion

In summary, we have developed an AIE-active probe with unique properties for biological imaging and sensing in cultured cells and in living systems. TPE-PyN₃ exhibits high specificity to mitochondria and can track the living cells for a long time with excellent photostability *in vitro*. *In vivo*, the high penetrability of TPE-PyN₃ enables all of the cells within the living zebrafish embryos to be brightly stained through simple incubation. The morphologies of cell populations could be clearly observed in real-time for dozens of hours and a high-resolution 3D visualization of the embryo was successfully reconstructed. Additionally, TPE-PyN₃ is sensitive to cell apoptosis in living zebrafish, which can potentially be used for *in vivo* drug screening. To the best of our knowledge, it is the first small-molecule AIE probe that can image and sense cell behaviors noninvasively in living systems.

All these merits make TPE-PyN₃ a promising candidate for studying behaviors of cells within living organisms in the fields of developmental biology, oncology, toxicology and drug discovery.

4. Experimental Section

Reagents and Materials: Fetal bovine serum (FBS), minimum essential medium (MEM), Dulbecco's modified eagle medium (DMEM), penicillin and streptomycin, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT), Sodium dodecyl sulfate (SDS), MitoTracker® Red FM, 5-chloromethylfluorescein diacetate (CellTracker™ Green, CMFDA), staurosporine were purchased from Invitrogen. Dimethylsulfoxide (DMSO), *N,N*-dimethylformamide (DMF), cadmium sulfate, Tricaine (ethyl 3-aminobenzoate) and all other reagents used in this study were obtained from Sigma-Aldrich and used as received without further purification.

Synthesis of TPE-PyN₃: 1-(4-Azidepropyl)-4-methylpyridinium hexafluorophosphate (TPE-PyN₃) was synthesized according to our previous study.^[24]

Cell Culture: HeLa or 3T3 cells were cultured in MEM containing 10% FBS at 37 °C with 5% CO₂. The medium contained 100 units/mL penicillin and 100 µg/mL streptomycin.

Cell Imaging: Cultured cells were seeded in a 35 mm petri dish with a glass cover slide. For mitochondrial imaging, cells were stained with 0.75 µM of TPE-PyN₃ for 5 min and 50 nM MitoTracker® Red FM (MT) for 15 min. In the coculture experiment, HeLa cells were stained with 3 µM of TPE-PyN₃ for 1 hour, followed by mixing with equal amount of unstained 3T3 cells and then cultured in a new dish. Cells were stained with 3 µM of TPE-PyN₃ for 1 hour or 10 µM CMFDA for 30 min (maximum working concentration and incubation time according to the protocol) for cell tracking and photostability assessment. Cells grown on the cover slide were washed three times with

phosphate buffered saline (PBS) buffer before imaging under a fluorescence microscope (Olympus BX41) or a laser scanning confocal microscope (Zeiss LSM7 DUO).

Cell Viability Evaluation: HeLa cells were grown in a 96 well-plate at density of 5000 cells per well. After overnight culture, medium with different concentrations of TPE-PyN₃ was added into the plate. After 1 hour incubation, 10 μ L of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT) solution (5 mg/mL in phosphate buffer solution) was added into the each well. After 4 hours incubation at 37 °C, 100 μ L of solution containing 10% SDS and 0.01 M HCl was added into each well. After 6 hours incubation at 37 °C, the absorbance of each wells at 570 nm was detected by a plate reader (Perkin-Elmer Victor3TM).

Toxicity Assessment of TPE-PyN₃ on zebrafish embryos: Living zebrafish embryos at 72-hour stage were reared in a 12-well plate at density of 12 per well for each concentration. Solution containing different concentrations of TPE-PyN₃ was added into the plate. After 2 hour treatment at 28 °C, embryos were washed three times with egg water and transfer to another plate for rearing at 28 °C. Control experiments were performed under the same conditions except replacing 10 μ M DMSO with TPE-PyN₃. Survival rate of each group was recorded every 24 hour after treatment. Fish was identified as dead if it was no longer moving and the heart was not beating.

Zebrafish Imaging: Living zebrafish embryos were incubated in solution containing 3 μ M TPE-PyN₃ for 40 min or 2 hour at room temperature for labeling study. In the apoptosis-induced study, living zebrafish embryos were stained with 3 μ M TPE-PyN₃ for 40 min, followed by treatment with 100 μ M cadmium sulfate or 1 μ M staurosporine for 5 hour at room temperature. Control group was kept under the same conditions except replacing 10 μ M DMSO with apoptosis-inducing chemicals. All zebrafish embryos were passed through three successive washes of buffer solution before observation. 0.01%-0.02% tricaine in egg water were used for anesthesia of fish before imaging.

Images were taken using a fluorescence microscope (Olympus BX41) or a laser scanning confocal microscope (Carl Zeiss LSM7 DUO). Three-dimensional data were analyzed and reconstructed by software ZEN 2012 (Carl Zeiss).

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Chart 1. Chemical structure of TPE-PyN₃.

Figure 1. Fluorescent images of HeLa cells stained with (A) TPE-PyN₃ (0.75 μ M) for 5 min and (B) MitoTracker red FM (MT, 50 nM) for 15 min. (C) Merged image of panels A and B. (D) Bright-field image of the cells. Excitation wavelength: 330–385 nm (for TPE-PyN₃) and 540–580 nm (for MT). Inset shows higher magnification of the mitochondria (boxed area).

Figure 2. Fluorescent images of TPE-PyN₃ or CellTracker™ Green CMFDA stained HeLa cells at different passages. Excitation wavelength: 330-385 nm (for TPE-PyN₃) and 460-490 nm (for CMFDA). Scale bar: 30 μm.

Figure 3. Fluorescent intensity of TPE-PyN₃ (red circle) or CellTracker™ CMFDA (blue square) stained HeLa cells with increasing number of sequential scans under confocal microscope. Emission signal was normalized to the maximum intensity at the beginning of irradiation.

Figure 4. Images of living zebrafish embryos stained with TPE-PyN₃. (A) Bright-field and (B) fluorescent images of the TPE-PyN₃ stained embryo. Top right, digitally zoomed inset. (C, F) Bright-field images of (C) upper half and (F) tail of the embryo. (D, G) Three-dimensional reconstructed confocal fluorescent images corresponding to the panels of C and F, respectively; scale bars represent the value of y axis. (E) Representative slices of views from different directions of the upper half of the stained embryo. (i) x-y plane; (ii) x-z plane; (iii) y-z plane. (H) Confocal image at high magnification.

Figure 5. Fluorescent images of TPE-PyN₃ stained living zebrafish embryos after (A) 12 hour, (B) 36 hour and (C) 60 hour. (D) Zebrafish embryo without staining. Scale bar: 30 μ m.

Figure 6. Tail rudiment of a living zebrafish embryo stained with TPE-PyN₃ in (A) control group, (B) cadmium sulfate or (C) staurosporine treated group. (D-F) The corresponding fluorescent images at higher magnification.

An AIE-active probe with distinctive properties for biological imaging and sensing in cultured cells and in living systems is presented. Noninvasive cell populations imaging, high-quality 3D tissue visualization, and cell apoptosis sensing *in vivo* are demonstrated as novel applications for studying behaviors of cells within living organisms.

fluorogen, aggregation-induced emission, mitochondria targeting, vital staining, cell apoptosis

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Supporting Information

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Figure S1. Cytotoxicity of TPE-PyN₃ on HeLa cells evaluated by MTT assay.

Figure S2. Fluorescent images of fibroblast 3T3 cells stained with (A) TPE-PyN₃ (0.75 μM) for 5 min and (B) MitoTracker red FM (MT, 50 nM) for 15 min. (C) Merged image of panels A and B. (D) Bright-field image of the cells. Excitation wavelength: 330-385 nm (for TPE-PyN₃) and 540-580 nm (for MT).

Figure S3. (A) Bright-field and (B) confocal images of the TPE-PyN₃ stained HeLa cells co-cultured with unstained 3T3 cells. (C) Merged image of panels A and B. Excitation wavelength: 405 nm; emission filter: 470-693 nm.

Figure S4. Confocal images of TPE-PyN₃ (3 μ M) and CellTracker™ Green CMFDA (10 μ M) stained living HeLa cells with increasing irradiation time. Excitation wavelength: 405 nm (for TPE-PyN₃) and 488 nm (for CMFDA); emission filter: 470-693 nm (for TPE-PyN₃) and 489-640 nm (for CMFDA); irradiation time: 11.31 s/scan. Scale bar: 20 μ m.

Figure S5. Survival rates of zebrafish embryos after staining of TPE-PyN₃ at different concentrations. Values are the mean of three replicates and error bars represent the standard deviation (SD).

Chart S1. Chemical structure of MitoTracker[®] Red FM.

Chart S2. Chemical structure of CellTracker[™] Green CMFDA.

Additional supporting movies

Fluorescent change of HeLa cells stained with TPE-PyN₃ (**movie 1**) and CMFDA (**movie 2**) under sequential irradiation. **Movie 3** shows representative 3D visualization of TPE-PyN₃ stained living zebrafish embryo.

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